



# Agilent Integrated Solutions for Design, Synthesis, and Quality Control of Guide RNA for CRISPR-Cas9 Genome Editing Workflows

## Application Note

Synthetic Biology

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### Abstract

The CRISPR-Cas9 system has rapidly been adopted for many genome engineering applications including creating gene knock-outs/knock-ins, genome mutagenesis, and gene activation and inhibition studies. This Application Note demonstrates how several Agilent products can be used in a CRISPR-Cas9 workflow, starting with the design of a guide RNA (gRNA) against a specific target, synthesis of the gRNA, quality control methods to ensure the integrity of the gRNA, and a Cas9 nuclease activity assay to test the efficacy of the generated gRNA. gRNAs were designed and synthesized against two target genes: ZFP42 and GUSB. The synthesized gRNAs were assessed for their overall quality and cleavage efficacy using the Agilent 2100 Bioanalyzer system.



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## Introduction

Genome engineering technologies are indispensable tools in several biotechnology applications that range from functional studies to developing better or novel biological systems<sup>1</sup>. One such tool, the clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated (Cas) protein system, is gaining interest due to the ease and versatility in editing the genome. The system consists of an RNA-guided nuclease (Cas9), and a guide RNA (gRNA) against a target sequence. The RNA-guided nuclease introduces a double-stranded break at the target sequence site if a protospacer adaptor motif (PAM) site is present adjacent to the target sequence. The double-stranded break can then be exploited to introduce gene knockdowns or point mutations.

These RNA-guided nucleases that the CRISPR-Cas systems are based on depend on simple complementary base pairing of a RNA-DNA duplex rather than complex protein-DNA interactions as in zinc finger nucleases (ZFN) or transcription activator-like effector nucleases (TALENs) techniques. The target specificity of the Cas9 nuclease can be modified by designing gRNAs to target different genes.

Designing a gRNA against a target gene is the first step in the CRISPR-Cas9 workflow. A unique target sequence, with minimal off-target homology to other sites, is desired. Agilent offers an integrated solution for designing and

synthesizing gRNAs, as well as a Cas9 nuclease assay to assess the efficacy of the synthesized gRNAs. This Application Note demonstrates the ease-of-use of Agilent SureDesign software for designing gRNAs, SureGuide gRNA synthesis kit for gRNA synthesis, and SureGuide Cas9 Programmable Nuclease kit for confirming the efficacy of the gRNAs. The Agilent 2100 Bioanalyzer system, with the Small RNA kit and a DNA analysis kit, helps in reliable quality control of the samples throughout the workflow.

## Materials and Methods

### Instruments, kits, and software

Agilent kits including Agilent DNA 7500 Kit (5067-1506) and Agilent Small RNA kit (5067-1548) for the 2100 Bioanalyzer system, the SureGuide CRISPR/Cas Complete Kit (5190-7714) comprising the SureGuide gRNA Synthesis Kit (5190-7719) and the SureGuide Cas9 Programmable Nuclease Kit (5190-7715), the StrataPrep PCR Purification Kit (400771) and Herculase II Fusion DNA Polymerase (600677), were used as recommended. The Agilent 2100 Bioanalyzer system (G2940CA) and the Agilent SureCycler 8800 (G8800A) were obtained from Agilent Technologies and used in accordance with the manufacturer guidelines. The Agilent SureDesign (<https://earray.chem.agilent.com/suredesign/>) web tool was used for designing gRNA sequences. Sequences designed were ordered and procured as lyophilized oligos from

Sigma-Aldrich. Human Genomic DNA was obtained from Promega and used as a template for PCR amplification.

### Guide RNA design

The gRNA sequences were designed using the SureGuide Wizard, part of the Agilent SureDesign web tool, against two genes: Zinc Finger Protein, Homology 42 (ZFP42) and Glucuronidase Beta (GUSB). The Wizard was configured to specify the target gene, target species, gene region, and mismatch requirements in target regions. The T7 promoter, crRNA, and tracrRNA sequences were added to the gRNA target sequences for the full length gRNA to be synthesized (Table 1) as per the user manual<sup>2</sup>.

In addition to the SureDesign Wizard outputs, three gRNA sequences were obtained from published resources<sup>3</sup> to design minimal and extended gRNAs. The published sequences were validated for their target specificity. The corresponding oligo templates required for the *in vitro* guide synthesis were procured from Sigma-Aldrich. Six gRNAs were designed for each gene: three minimal and three extended design variants. The gRNA designs were designated as: M: minimal design, VM: validated minimal design, E: extended design, and VE: validated extended design along with the gene name, where M and E are the sequences designed by the SureDesign software, and VM and VE are designs based on the published and validated sequences.

Table 1. Oligo templates required for the synthesis of extended and minimal design gRNA using the gRNA synthesis kit. The example shown includes a GUSB target sequence.

Extended design gRNA framework	Forward primer	5' CG ATG TAA TAC GAC TCA CTA TAG <b>GTT AGA ACC AGT CTG GAG CTA</b> <b>GTT TTA GAG CTA TGC TGA</b> AA 3'
	Reverse primer	5' <b>AAG CAC CGA CTC GGT GCC ACT TTT TCA AGT TGA TAA CGG ACT AGC CTT ATT TTA ACT TGC TAT GCT TTT CAG CAT AGC TCT AAA ACT</b> 3'
Minimal design gRNA framework	Oligonucleotide sequence	5' <b>CGG ACT AGC CTT ATT TTA ACT TGC TAT TTC TAG CTC TAA AAC TAG CTC CAG ACT GGT TCT AA CC</b> TA TAG TGA GTC GTA TTA CAT CG 3'

Legend: tracrRNA transcript; crRNA transcript; 20 nt target sequence, T7 polymerase initiator nucleotides, T7 promoter sequence

## Guide RNA synthesis

Figure 1 presents the overall workflow as a schematic diagram. The *in vitro* guide synthesis was carried out using the SureGuide gRNA synthesis kit following the manufacturer guidelines. For the extended design gRNA, a longer DNA template was required than could easily be synthesized in a standard oligo synthesis reaction. To generate the longer template, two standard length oligos were paired at the ends, and a fill-in reaction was carried out using Agilent Herculase II polymerase.

For the minimal gRNA variants, a master mix constituting 5x transcription buffer, rNTPs, DTT, T7 polymerase, RNase block, T7 promoter primer, and yeast pyrophosphate was made and mixed with oligo templates. The mixtures were incubated at 37 °C for 1 hour to transcribe the gRNA, followed by a 15-minute incubation with a DNase enzyme to remove the DNA template. The extended design synthesis reaction follows the

same procedure, but without the T7 promoter primer in the master mix as the double-stranded template does not require priming. The transcribed gRNAs were then purified using the gRNA purification kit, and were stored at -80 °C until use.

## Guide RNA quality control

The purified gRNA samples were analyzed in triplicate using the Agilent Small RNA kit with the Agilent 2100 Bioanalyzer system. The gRNA samples were diluted to the assay quantitation range, denatured at 80 °C for 2 minutes (to minimize secondary structure), and analyzed following the manufacturer guidelines<sup>4</sup>.

## Cas9 Nuclease assay

Forward and reverse primers were designed using the Primer-BLAST<sup>5</sup> to PCR amplify a gene region that contains the genomic target sequences. The primers were procured from Sigma-Aldrich as

lyophilized oligos and reconstituted in TE buffer. A PCR reaction was prepared containing 250 nM of each primer, and 350 ng of human genomic DNA in a 50 µL reaction volume. PCR cycling parameters were: 95 °C for 2 minutes, followed by 30 cycles of 95 °C for 10 seconds, 61–65 °C for 20 seconds, and 72 °C for 150 seconds. A final extension of 72 °C for 3 minutes was used to fill in any protruding ends. The amplicons were purified using the StrataPrep PCR purification kit<sup>6</sup>. The amplicons were mixed with 10x Cas9 digestion buffer, DNA templates, and Cas9 nuclease enzyme in the presence or absence of different gRNAs. The mixtures were incubated at 30 °C for 30 minutes, followed by an additional 15 minutes at 65 °C to inactivate the Cas9 nuclease<sup>7</sup>. A control reaction was also carried out using the supplied control gRNA and control DNA template. The digestion products were analyzed using the DNA 7500 kit with the 2100 Bioanalyzer system as per the manufacturer's guidelines.

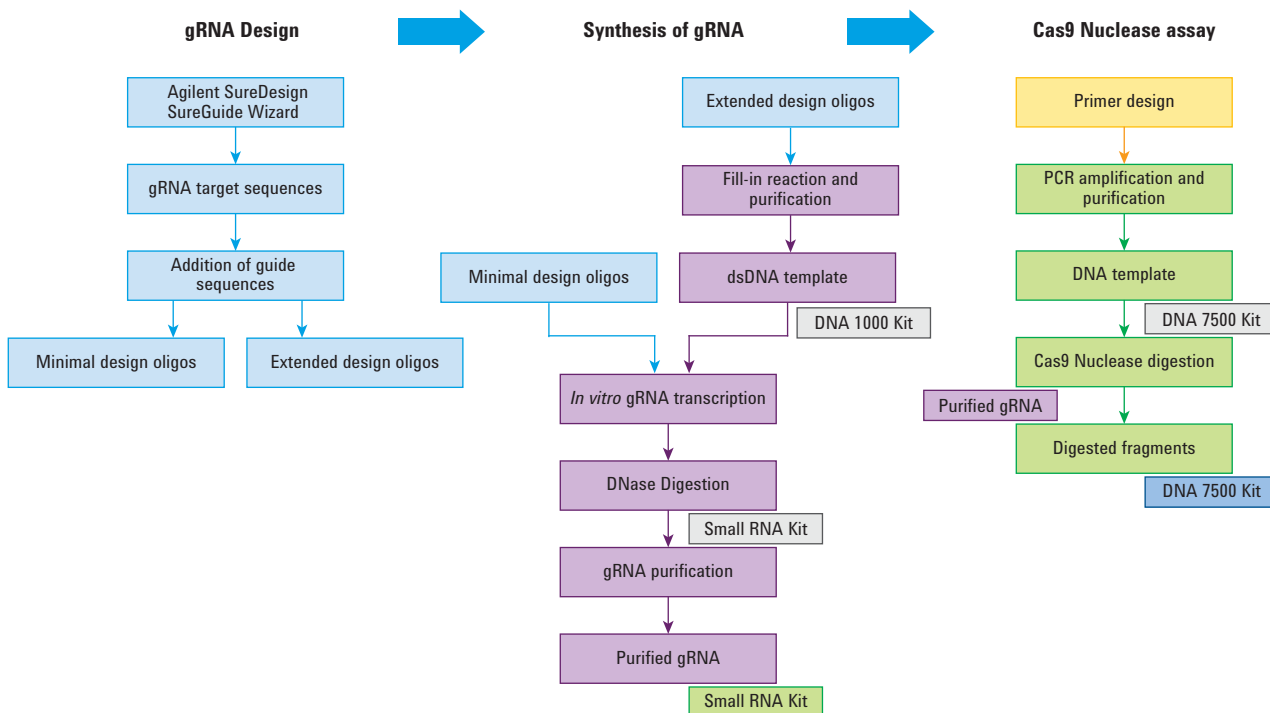


Figure 1. Schematic diagram showing gRNA design, synthesis, and Cas9 assay workflow (gray boxes indicate optional steps).

## Results and Discussion

### Design of Guide RNA

Several gRNA sequences were designed using the SureDesign SureGuide Wizard, an online program. The target sequences were defined using the gene name or ID. Alternatively, users can upload a custom DNA sequence for gRNA design. Parameters such as the Protospacer Adjacent Motif (PAM) sequence can be selected based on the Cas9 protein used. NGG was chosen as the PAM sequence for this workflow since the Agilent kit uses Cas9 from *Streptococcus pyogenes*. Users can also input custom PAM sequences if different Cas proteins are being used. The Wizard can also be configured to search for gRNA sequences that start with two G bases at the

5' end, which is required for the *in vitro* T7 polymerase transcription, or the Wizard can append the GG bases to any gRNA sequence generated.

Figure 2 presents a typical output from the SureGuide Wizard that shows the gRNA sequence, rank, hits, and position within a genome map. gRNA sequences can then be hand-picked from the list, and synthetic DNA oligo templates can be derived by adding the T7 promoter sequence, crRNA sequence, or tracrRNA sequence in the proper format for either the extended design or minimal design. It is advised to design more than one gRNA sequence against a target, since the functionality of any given gRNA varies, and needs to be experimentally confirmed.

### Quality control of guide RNA

The transcribed and purified gRNAs were analyzed using the Agilent Small RNA kit with the 2100 Bioanalyzer system to assess the size, purity, and quantitation of each gRNA. Each gRNA sample was analyzed in triplicate (example in Figure 3A). A representative profile for each gRNA sample is presented in the gel image (Figure 3B), each showing an intense predominant peak with an average size of 60 nucleotides (nt), similar to the control gRNA.

Figure 3C shows a similar gel image for the extended design gRNA samples. The extended design gRNA samples migrated around 90 nt, as expected.

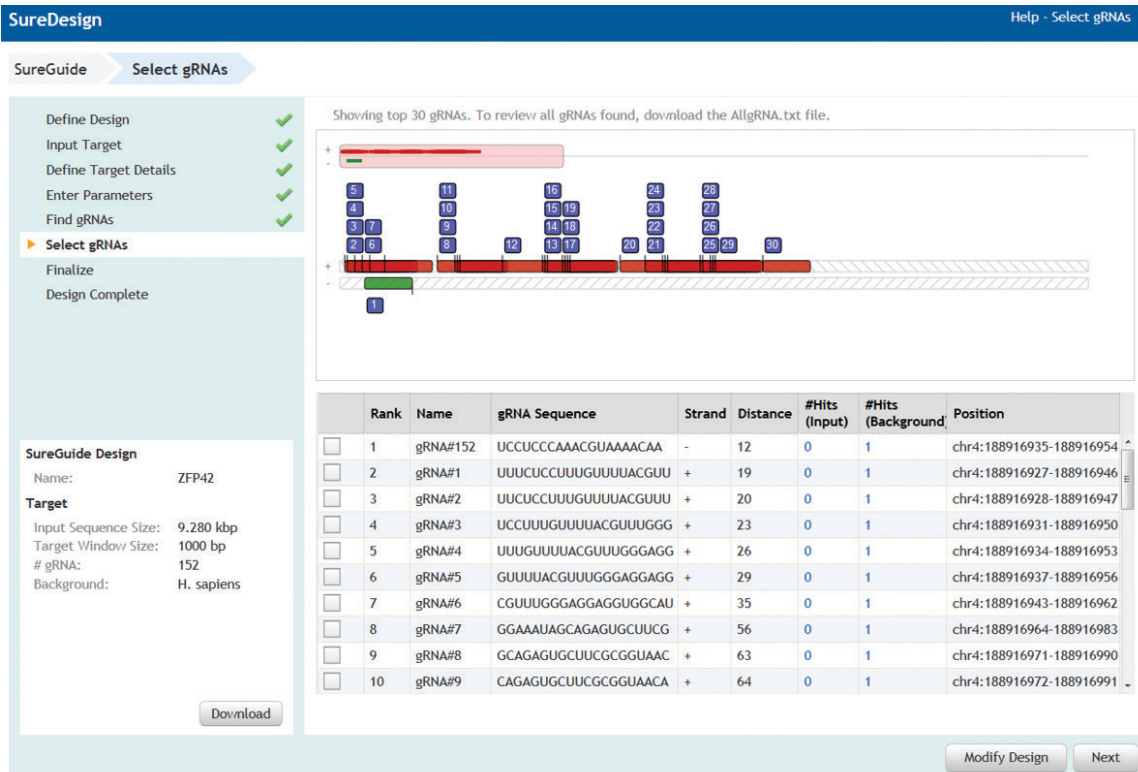


Figure 2. SureGuide Wizard output window showing the list of gRNA sequences ordered by rank.

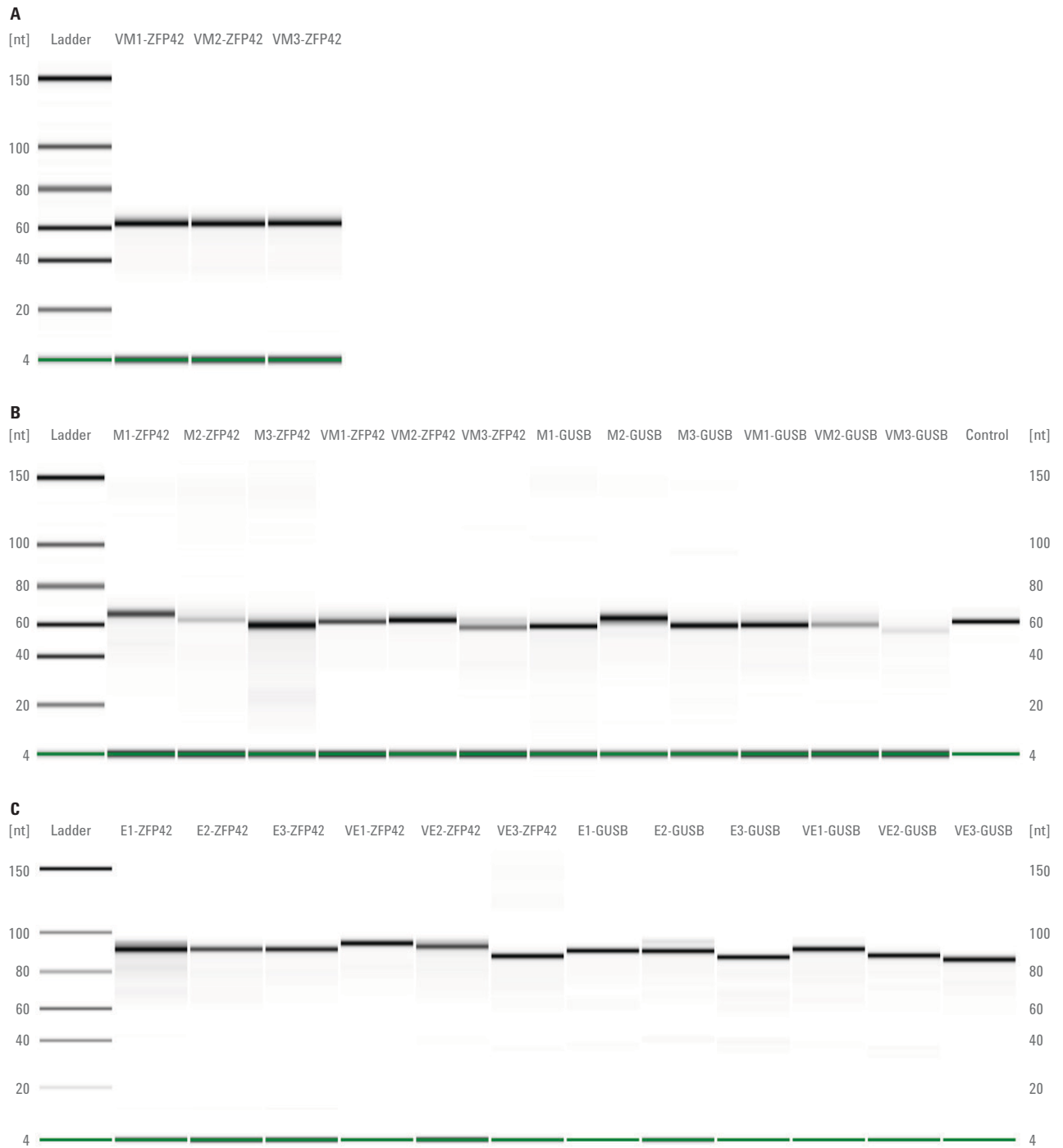


Figure 3. A) Triplicate analysis of a gRNA sample using the Small RNA kit. B) Gel-like image showing minimal design gRNA sample peaks around 60 nt. C) Gel image showing extended design gRNA sample migrating around 90 nt.

The 2100 Bioanalyzer system demonstrated excellent reproducibility in determining the sizes of the gRNA samples. The sizing precision CV for the gRNA samples was found to be 1 % or better, as presented in Figure 4A. The samples were also quantified using the Agilent Bioanalyzer 2100 Expert software, and those data are presented in Figure 4B. The quantitation precision CV was 13 % or better, which is within the kit specification of 25 %. The data shown in Figure 4 demonstrate that gRNA samples can reliably be assessed using the Small RNA kit and the 2100 Bioanalyzer system.

### Cas9 Nuclease assay

The efficacy of the synthesized gRNAs was evaluated using the Agilent SureGuide Cas9 Nuclease kit. The genomic region against which the gRNA was designed was amplified in such a way that Cas9 digestion resulted in two fragments of different sizes. The fragments were analyzed using the DNA 7500 kit with the 2100 Bioanalyzer system to check for gRNA efficacy.

The cleavage efficiencies of gRNAs with the same target sequence, but with either the minimal or extended design, can vary due to the length of the backbone. The extended design gRNAs show better Cas9 affinity, resulting in increased cleavage of the DNA. However, the minimal design is preferred in some scenarios to reduce

the cost and complexity of synthesizing a longer oligo template. Figure 5 illustrates the nuclease assay using the gRNA against the GUSB. The gel image shows an amplicon that is cleaved into two fragments upon digestion with Cas9 containing different target gRNA sequences. The assay highlights the difference in target affinity between the minimal design and the extended design. For example, the minimal design gRNA M3-GUSB failed to cleave the template, whereas the extended design resulted in template cleavage. Similarly, the M2-GUSB gRNA leaves a greater fraction of uncleaved DNA template when compared to the E2-GUSB version. The positive control gRNA showed the expected fragment sizes, and the negative control showed no cleavage.

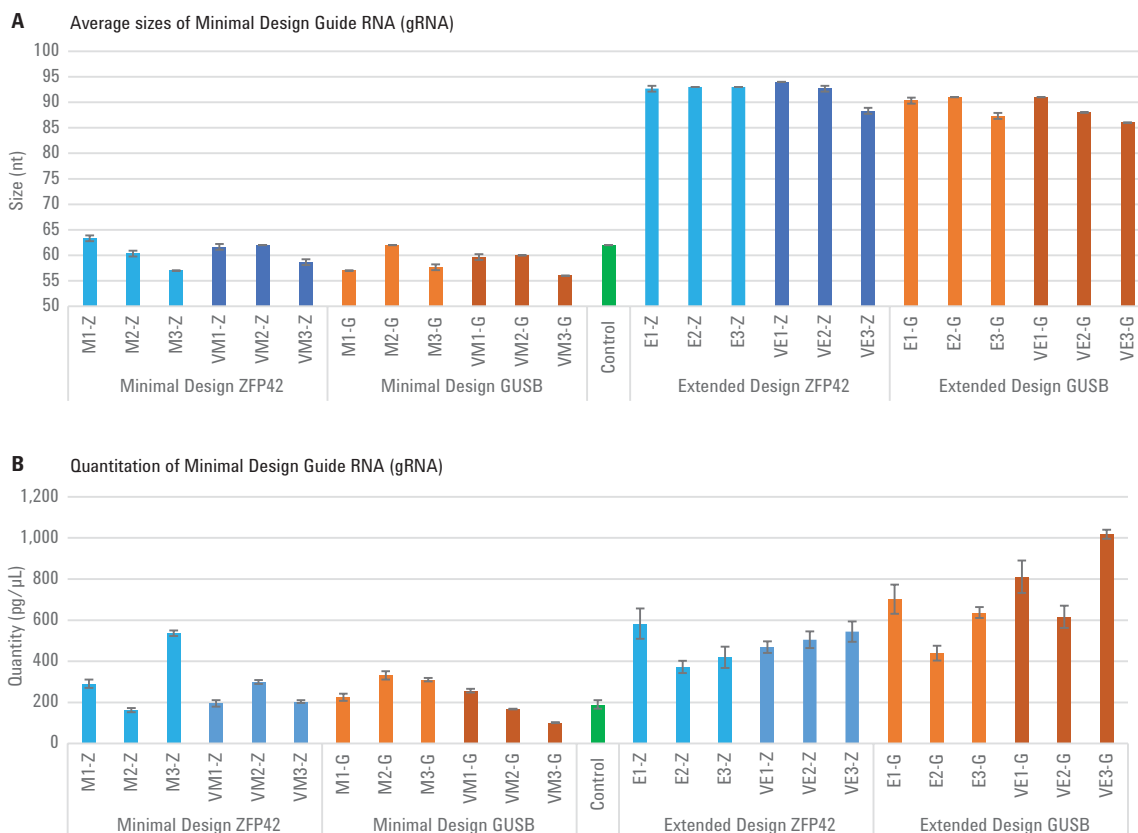


Figure 4. A) Sizing precision of an Agilent 2100 Bioanalyzer Small RNA kit in analyzing gRNA samples (note: the x-axis scale is adjusted to show error bars). B) gRNA sample quantitation using a 2100 Bioanalyzer Small RNA kit.

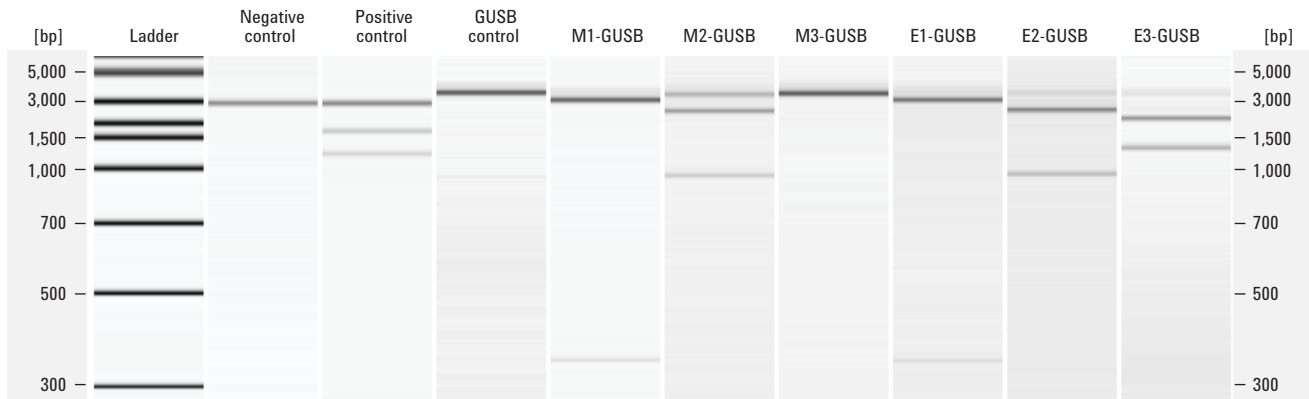


Figure 5. DNA 7500 gel image of the Cas9 nuclease assay for minimal and extended design gRNAs against the GUSB gene.

The efficiency of the gRNA can also be expressed as cleavage percentage, calculated using Equation 1. Cleavage percentage provides a metric to compare the efficiency of different gRNA designs against a given target.

$$\text{Cleavage \%} = \frac{\text{Sum of peak area of cleaved fragments}}{\text{Sum of peak areas of cleaved and uncleaved peaks}} \times 100$$

Equation 1. Cleavage efficiency calculation

The electropherogram can be used to obtain the area of each peak to calculate the cleavage efficiency, as presented in Figure 6. Table 2 summarizes the results of a Cas9 nuclease assay for several gRNAs with the same target sequence in both minimal and extended design formats against ZFP42 and GUSB. The data clearly show a higher cleavage efficiency for the extended designs over the corresponding minimal design. gRNA samples may be chosen for further experiments based on cleavage rates.

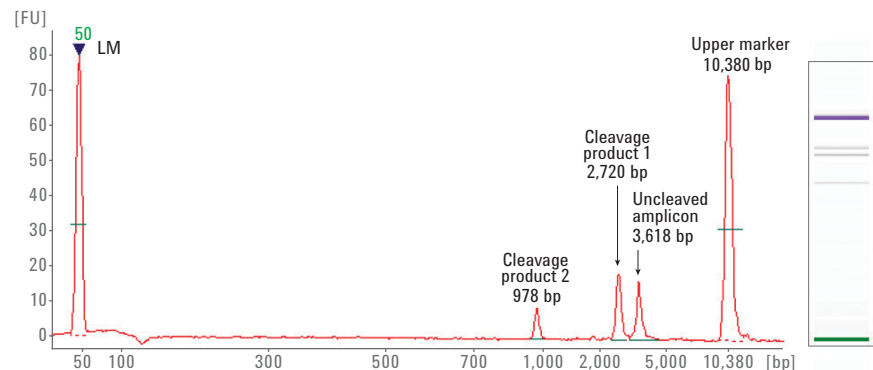


Figure 6. Electropherogram showing the Cas9 nuclease assay using M2-GUSB gRNA. The cleaved products and the intact amplicon are shown with the corresponding gel image.

Table 2. Cas9 Cleavage efficiency of the gRNA used in the study.

Target gene	Amplicon size (bp)	gRNA design	Minimal design gRNA		Extended design gRNA	
			Cas9 Cleaved fragments (bp)	Cleavage efficiency %	Cas9 Cleaved fragments (bp)	Cleavage efficiency %
ZFP42	3,621	Control	No cleavage	n/a	No cleavage	n/a
		Design 1	No cleavage	0 %	2958, 666	91.1 %
		Design 2	No cleavage	0 %	3492, 78	83.6 %
		Design 3	No cleavage	0 %	3372, 83	81.3 %
GUSB	3,638	Control	No cleavage	n/a	No cleavage	n/a
		Design 1	3236, 358	92.4 %	3116, 356	97.6 %
		Design 2	2720, 976	66.3 %	2677, 976	90.9 %
		Design 3	No cleavage	0 %	2320, 1361	91.1 %



## Troubleshooting steps

In addition to the required quality control steps and efficacy assessment, the 2100 Bioanalyzer system can also be used to troubleshoot intermediate steps during the workflow, as indicated by the gray boxes in the schematic diagram in Figure 1. A few of the intermediate steps include:

### Guide RNA purification

The Small RNA Kit can be used to check for successful transcription before the final purification step. This would allow the user to optimize the input oligo concentration, yield, and incubation times, which may vary between different sequence designs.

### Extended design fill-in reaction

The extended design gRNA synthesis requires a fill-in reaction using a forward and reverse primer followed by purification of the double-stranded template. A quick quality check using the DNA 1000 Kit helps users verify the presence of successful fill-in products with an average size of 130 bp. Additional peaks could indicate truncated primers or other contaminants in the reaction. A DNA 1000 quality check of the fill-in products will help in further optimization of the reaction before *in vitro* transcription.

### PCR amplification of the DNA template

To check the gRNA specificity, a 3–15 kb DNA template containing the target gRNA sequence is required to perform the Cas9 nuclease assay. PCR amplification is performed to amplify the target DNA region using a high-fidelity enzyme, for example, Agilent Herculase II fusion DNA polymerase, that supports longer targets. The resulting PCR amplicon can then be analyzed using the DNA 7500 kit to check for correct size and yield before the Cas9 nuclease assay.

## Conclusion

- Agilent SureDesign and the SureGuide Wizard simplifies gRNA design for *in vitro* site-specific cleavage of dsDNA.
- Designed gRNAs were successfully prepared using the Agilent SureGuide gRNA synthesis kit.
- The Agilent 2100 Bioanalyzer system with the Small RNA kit provides high-resolution analysis of small RNA fragments allowing accurate and reproducible analysis of gRNAs, providing information on size and yield.
- The Agilent SureGuide Cas9 nuclease assay can be used to measure the cleavage efficacy of synthesized gRNA molecules *in vitro*.
- The Agilent 2100 Bioanalyzer system can be used in the screening of the nuclease assay, providing visual and digital assessment for cleavage.
- This study demonstrates the seamless integration of Agilent solutions for gRNA design, *in vitro* RNA synthesis, and confirmation of *in vitro* cleavage efficiency.

## References

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